[redacted]

[redacted]

21p

(NASA CR 55184)

National Aeronautics and Space Administration

(NASA Contract NASw-747)

Renewal Proposal No. 160 and Quarterly Status Report No. 2

For

Research in Photosynthesis

[6]

Proposal #160 December, 1963

Martin Co., 5524004

RIAS
7212 Bellona Avenue
Baltimore 12, Maryland

Principal Investigator: Bessel Kok

Marketing Manager: J. S. Fly, Jr.

National Aeronautics and Space Administration

Contract NASw-747

Renewal Proposal and Quarterly Status Report No. 2

Research in Photosynthesis

Summary

In a fair sized group such as ours several projects are generally going on simultaneously. Some of these extend over a long time and may be pursued intermittently while others may yield a rather immediate and clear-cut answer. It therefore may be difficult to evaluate the relative significance of the various programs described in this report. This brief introduction might serve to put things in perspective.

A few projects came to fruition during the last year. The first of these concerns the peculiar metabolic path of lipid synthesis by cell free Euglena extracts studied by Dr. Cheniae. Two publications reporting his work are in the press, two more are coming forth. Another such project concerns the photo-oxidation of cytochrome c, cytochrome f, and plastocyanin by chloroplasts treated with detergent or other means so that only one of the two photo-reactions is surviving. This work has resulted in one paper in the press and one more to come. A third subject which has been studied extensively is the respiration during photosynthesis, specifically the exchange of oxygen. This study, for which a special mass spectrometer technique was developed, has resulted in two significant papers with one or two more in preparation. In the foreseeable future we will put more emphasis on the carbon dioxide part of this problem since preliminary data have shown that there is no simple relation between oxygen uptake and output and carbon dioxide output and uptake. A fourth project which was brought to a reasonable end point concerned the activation spectra of the

reduction of pyridine nucleotide by chloroplasts. A publication concerning this work appeared recently. A related project consisted of the measurement of activation spectra of photophosphorylation which was run concurrently with the measurements of TPN reduction but which yielded much more complex results. Also the results of this study will be published soon. Our quantitative studies of the fast photochemical EPR signal made in collaboration with Dr. Beinert have been summarized recently in a final paper.

Some other projects are presently in the focus of attention:

Because of difficulties encountered in the purification and isolation of photocatalyst P700 from abundant sources such as spinach leaves or normal algae, future efforts might include different approaches, for instance, using artificially produced algal mutants. At present promising results are being obtained in the continued studies on the two photoreactions of photosynthesis and the site of phosphorylation. One paper on this subject was published this year; another is in press. Considerable work has been done (and presented in a brief paper) during the last year on the formation of an ATP hydrolyzing enzyme which is made by light in chloroplasts. This work is still in progress and now includes the peculiar changes of chloroplast structure accompanying the process. Another project on which much time is being spent concerns the fluorescence of photosynthetic material. Especially in our low temperature studies we have observed several interesting new phenomena. Preliminary reports on these data have been published.

Studies on the inhibitory effects of strong visible light upon the photosynthetic apparatus are being pursued further in the hope of elucidating the underlying mechanism.

FUTURE RESEARCH PLANS

We plan a continuation of our present research program concerning the light transformations in photosynthesis. The progress reports continued in the next section may help to elucidate this program, though emphasis and direction may change depending upon future progress.

In general our program is directed towards an analysis of the sensitization and interplay between the two photoreactions, the chain of oxidation-reduction steps and the locus of photophosphorylation. More specifically our program includes the following topics:

(1) The photoactivation of ATPase activity and its associated effects upon the structural properties of the chloroplast;

(2) Studies of fluorescence emission and rates of chloroplast reactions in flashing illumination which are aimed at analyzing the photosynthetic unit and the manner by which absorbed light is focussed into the reaction centers of the photoacts;

(3) Response of the photosynthetic apparatus to strong light i.e. the mechanisms responsible for rate saturation and for photoinhibition;

(4) Further characterization of the chlorophyll complex P700 and its mode of operation in the electron transport chain;

(5) Analysis of the oxygen liberating system by kinetic studies of net rate and isotopic exchange;

(6) Clarification of the requirement of CO_2 for chloroplast reactions and its possible implications for the primary reduction process in whole cells;

(7) Interactions between respiration and photosynthesis, possibly including the mechanisms by which (chloroplast) photosynthesis decelerates and accelerates (cytoplasmic or mitochondrial) respiration.

PROGRESS REPORT

I. Effects of Strong Light on Photosynthesis

One of our programs deals with the problems encountered in cultivating algae (as well as higher plants) in strong light. Progress in this area should allow smaller and more efficient algal cultures for space flight both with solar and artificial illumination. Strong light is used inefficiently because presently available algae show a relatively low light saturation level and are moreover prone to photoinhibition. So far our studies have been concerned with the kinetics and the mechanism of photoinhibition observed with isolated chloroplasts. This material allows a greater variation of reaction conditions than whole algae.

Inhibition by strong visible light is very similar to that by ultraviolet radiation, although an accurate comparison of the effectiveness of the two wavelength areas still is awaiting.

Exposure to strong light proved to inactivate all normal photochemical activities of fresh chloroplasts including photophosphorylation and the reduction of TPN in the p' esence of reduced indophenol. This would indicate that both photoreactions are destroyed by photoinhibition. However, after detergent treatment of photoinhibited material one can still observe the photo-oxidation of cytochrome c and a considerable fraction of P700 which would indicate that the "first" photosystem is still intact. One of the most typical effects of photoinhibition is a decrease of fluorescence yield which suggests that light is drained into the trapping centers but not converted. So far, we have been unable to obtain a satisfactory explanation for these observations. The peculiarity of the phenomena certainly warrants further studies.

II. Lipid Metabolism

A. Investigations on lipid metabolism in photosynthetic organisms is being terminated. Studies on fatty acid synthesis with extracts of Euglena have reached a point where the major conclusions of the original supposition have been attained. One manuscript is in press (Biochim Biophys Acta), a second has been submitted (Arch. Biochem) and two more are near completion. The major conclusion that has been reached is that long-chain fatty acid synthesis in Euglena operates by a mechanism different from any reported to date. The evidence to support this conclusion is partially summarized as follows:

1) Fatty acids, predominantly saturated of 10-20 carbon atoms, are formed from acetyl-1-C^{14}-CoA with 60 to 70 per cent of the radioactivity associated with fatty acids of 16-20 carbon atoms;
2) These acids are formed by a de novo process as indicated by results of chemical degradations on the acids;
3) Requirements for synthesis from acetyl-1-C^{14}-CoA include ATP, DPNH, TPNH and a divalent cation;
4) Stoichiometric studies indicate that for each acetyl unit incorporated into long-chain fatty acids one ATP and two reduced pyridine nucleotides are required. This stoichiometry is similar to that obtained for the malonyl-CoA pathway. The following experiments to implicate the malonyl-CoA pathway have all been negative: (1) requirement for HCO_3^-; (2) inhibition by avidin; (3) demonstration of

acetyl-CoA carboxylase activity; and (4) incorporation of malonyl-1,3-C^{14}-CoA.

6) To explain these results a mechanism involving the phosphoenolate of acetyl-CoA has been proposed. Some theoretical supporting evidence for such a compound is found in the literature. Our experimental evidence for the compound is suggestive.

B. In addition to these studies on fatty acid synthesis with extracts of Euglena we have continued our investigations on the unique sulfolipid of photosynthetic tissue. Kinetic studies on incorporation of $S^{34}O_4^=$ into cellular material, including sulfolipid, have revealed that in 15-second to 30-minute exposure of Chlorella to $S^{35}O_4^=$ under photosynthetic conditions, the radioactivity in the lipid fraction constitutes 25 to 50 per cent of the total amount of S^{35} accumulated in alcohol soluble fractions. Examination of various fractions by chromatographic and electrophoretic techniques have given no indication that such compounds as (1) sulfolactaldehyde, (2) 6 - sulfoquinovose, or its glycoside are intermediates in the biosynthesis of the sulfolipid. This is in contrast to observations made by Shibuya et al. (Studies on Micro Algae and Photosynthetic Bacteria, p. 627, Univ. of Tokyo Press, 1963). These mentioned compounds do not appear except after long (12-20 hours) labeling periods and probably represent metabolism of (1) cysteic acid and (2) sulfolipid itself.

A great number of conditions have been tried and varied in an attempt to trap possible intermediates between $SO_4^=$ and sulfolipid. We have occasionally detected a compound with properties of a nucleoside sugar sulfonate. This compound was suggested to be a key intermediate in the original proposal. Other workers (Shibuya, et al.), at our suggestion, have also detected this compound but have been similarly unsuccessful to date in implicating this compound in the pathway of sulfolipid biosynthesis.

The fact that we have been unable to routinely trap this or other intermediates suggests that the sulfonic group may be introduced directly into lipid by condensation between active sulfate or sulfite and an anhydroglycolipid, or between sulfite and a glycolipid with an active hydrogen followed by oxidation. Experiments to test these hypotheses are being pursued.

C. Results on $P^{32}O_4^{\equiv}$ incorporation into phosphatides strongly suggest a sequence of phosphatide biosynthesis similar to that demonstrated in animal tissues. Labeling of intermediates with C^{14} is in progress to confirm and substantiate the findings obtained with P^{32} labeled intermediates.

III. Mechanism of Photosynthetic Phosphorylation

Chloroplasts prepared and assayed under conditions which yield good rates of photosynthetic phosphorylation have negligible ATPase activity. The apparent irreversibility of the reaction, even though favored by a large free energy change, has been a handicap in studies on its mechanism. Petrack and Lipmann ("Light and Life", McElroy and Glass, ed., Johns Hopkins Press, Baltimore, 1960) have induced a light dependent hydrolysis of ATP in chloroplasts by including sulfhydryl compounds in the reaction system. We have shown that this "photohydrolysis" is actually a photoactivation of ATPase activity. The conditions required for development of the activity are low light intensities and the presence of -SH compounds. The requirements for development of ATPase are mild and thus the reaction may conceivably occur in vivo. The dephosphorylation has parameters which are both similar to, and opposite from, photosynthetic phosphorylation and hence undoubtedly reflect a reversal of at least a portion of the phosphorylation sequence.

The overall pathway leading to ATPase activity is complex, since activation and hydrolysis are separate reactions and respond differently to various treatments. For instance, the activation of hydrolytic activity is inhibited by ammonia (an uncoupler of photosynthetic phosphorylation) but the hydrolysis rate is enhanced by the same compound. Our results may be summarized as follows: Two ATPase activities may be developed with FMN (I) or ATP (II) in the light. System I decays much more rapidly in the dark and may be reactivated by additional exposure. System II is enhanced by ammonia given in the dark: system I

is inhibited. Both systems are destroyed by sonic oscillation or treatment with detergents. System I is activated by short wavelength light, in common with FMN mediated phosphorylation, while system II is long wavelength activated.

IV. Interaction of Respiration and Photosynthesis

The cellular processes of photosynthesis and respiration have generally been presumed to act independently. Conversion of carbon dioxide to starch has been accepted as the photosynthetic process and the catabolism of this starch as the respiratory energy supply for cell growth. Elaboration of the intermediates in carbon dioxide reduction and the reducing power for this process pointed up the fact that not one of these compounds was unique for photosynthesis, but that all intermediates in photosynthesis are also intermediate compounds in respiration. Complete separation of the two processes, even though they occur in different organelles within the cell, would therefore appear unlikely. A well known example of metabolic interaction is the Pasteur effect.

The first evidence for such an interaction between photosynthesis and respiration came from the work of Weigel, et al. [J. Am. Chem. Soc. 73, 5058 (1951)] and Brown and Weiss [Plant Physiol. 34, 224 (1959)] who found CO_2 production to be inhibited during illumination. We have examined the effect of illumination on oxygen uptake and production in green and blue-green algae using a sensitive mass spectrometric method. The results showed two effects--i.e., light either decreased or increased oxygen uptake depending upon the wavelength of exciting light. If the light was absorbed primarily by chlorophyll a, uptake was diminished; if absorption was by accessory pigments, oxygen uptake increases. However, in both cases CO_2 production decreased. The analogy to the Pasteur effect is very good:

Photosynthesis is to respiration as respiration is to fermentation.

The response of oxygen uptake to mixed light beams of various colors and to inhibitors has been studied to determine the mechanism by which this oxidation reaction occurs. For instance, addition of suppressor light (chlorophyll a_1) results in a decrease in accelerated oxygen uptake caused by accessory pigment illumination. These results indicate both suppression and acceleration result from an excess of one or the other photoact. They also provide an interpretation of Emerson's observation that "enhancement" was sometimes negative.

Present work is directed toward understanding the mechanism of photosynthetic control of respiration.

A recent publication, describing the special mass spectrometric method used in these studies, may be found in Hoch and Kok [Archives of Biochemistry and Biophysics 101, 160-170 (1963)].

V. Studies of Electron Para Magnetic Resonance Signals

We have continued measurements of EPR signals in photosynthetic tissue in collaboration with Dr. H. Beinert. This work began about a year ago. We have attempted firstly to identify the photochemically-induced spin signal with our photocatalyst P700. The kinetics of the formation of these spin signals did rather precisely match the expectation following this hypothesis. Secondly, the much more difficult task was to make quantitative measurements of the maximum number of spins per number of chlorophyll molecules in the sample. Absolute measurements of the spin signals are not easy, and we have collected a number of data at several different occasions with live material chloroplasts or preparations enriched in P700. The last series of measurements was done with two instruments, one in the Kettering Foundation, Yellow Springs, Ohio, the other in Wisconsin. Accuracy and consistency seems now great enough to warrant a final paper which should follow the two notes published before. The result can be summarized that we feel that there is a definite correlation between P700 and the photo-induced spin signal. The ratio between these is 0.3-0.5 and we are pondering the possible significance of this ratio. Either we have assumed the wrong molar extinction for P700 (we used the same extinction as that of chlorophyll and there is no necessity to adhere to this), or a more intriguing possibility is that upon photo-oxidation of P700 not one, but two spin signals are generated--one by the "hole", the other by the "electron". However, this is entirely speculation and measurements of another type have been designed to decide.

VI. The Role of Cytochrome f and of Plastocyanin in Photosynthesis

Chloroplasts which have been treated with detergent or have been extracted with aqueous acetone will no longer evolve oxygen or sustain a net rate of dye or TPN photoreductions. They will, however, photo-oxidize a number of materials, one of which is cytochrome c as was found first by Vennesland and co-workers. We have studied this process in some detail. We measured the activation spectra which indicated "long wave" chlorophyll as the sensitiser. We also found evidence that $P700^+$ is the primary donor of the electron to cytochrome c. We observed that cytochrome c oxidation is stimulated by the addition of a low potential redox agent such as methyl viologen. More intriguing was that our purification of what Vennesland et al. had called a "cytochrome c photo-oxidase" revealed that this enzyme was identical with "plastocyanin," a copper containing enzyme recently isolated from chloroplast by Katoh. In the presence of plastocyanin, cytochrome c photo-oxidation can proceed at rates much faster than normal reduction rates in chloroplast reactions. We found that cytochrome f (prepared from Euglena) was nearly as efficient a mediator. In the presence of such a catalyst the quantum yield of this oxidation could approach 1. Other kinetic features of this system appeared interesting. The temperature effect proved to be very small indeed, which might indicate that the rate limiting step should be sought in a charge transfer reaction between two components on the chloroplast matrix viz. P700 and cytochrome f. The

same was indicated by the observation that the quantum yield of the photo-oxidation of cytochrome f (used as a substrate rather than a catalyst) was directly proportional to the redox state of cytochrome f in the system. We feel these studies, to appear soon in press, have yielded more insight in the first photoreaction of photosynthesis. Points which need further elucidation are the stimulation by flavin or viologen and the role of plastocyanin in complete photosynthesis. We cannot help wondering about the analogy between cytochrome oxidase in respiration and the high potential heme and copper enzymes (cyt. f and plastocyanin) in photosynthesis.

VII. Electron Transport, Interaction Between the Two Photoacts

Using sensitive difference spectroscopy, we have pursued our experiments about the loci where the primary oxido-reductions in photosynthesis take place. The presently most popular theory assumes formation of two photo-reductants made by the two photo-acts, one of a medium and the other of a low redox potential. Of course, a low potential reductant must be formed, otherwise we would never observe the reduction of TPN, viologen, etc. Also a medium potential reductant must be formed, or we would not see reduction of P700 by the second photoact. The question is, can this medium potential reductant ($E_o^1 < +0.43$ volt) operate with external substrates. It actually has been proposed, (Witt, Arnon), that indophenol dye is reduced by this hypothetical weak reductant. We have been able to show that at least under normal conditions reduction of this dye occurs via the strong reductant of the first photoact. Experiments made in long wavelength light (which does not sensitize the second photoact) and in the presence of the poison DCMU (which kills the second photoact) showed that the dye is still reduced with good yield and rate. These observations together with studies of other chloroplast oxidants may have further consequences. One of these concerns the site of photophosphorylation. They point to the possibility that this locus is at the substrate (low potential) level rather than, as is generally assumed, at the cytochrome (b-f) level. At this moment we are trying to design more decisive experiments concerning this point. Quite interesting results are being obtained in experiments with a mutant of the green algae, Scenedesmus. While visiting us for some joint mass spectrometer experiments, Dr. Norman Bishop (Florida State University) brought along

this mutant (#8) which is incapable of reducing carbon dioxide or evolving hydrogen but can still reduce benzoquinone with the concomitant evolution of oxygen. We have investigated this mutant and found that it did not show any turnover of P700. This is consistent with Dr. E. Weaver's finding that this mutant does not show the electron spin signal which we have identified with P700. Our various other measurements with this peculiar mutant concerning its fluorescence and absorption properties may result in a brief paper.

So far we have been rather unsuccessful in our attempts to elucidate the role of cytochromes in photosynthetic electron transport. Either their role is limited or (these intermediates) (cyt. f) react so fast that they escape our present detection method (time resolution 10^{-3} sec.). Our new difference apparatus, which was completed last summer, has many technical features not shown by our earlier equipment, but some of these cannot yet be fully utilized until we further improve the signal-to-noise ratio. A more serious limitation of our present equipment, which utilizes mechanical light shutters, rests in the tremendous velocity with which the initial photoprocesses appear to occur. We are presently designing a method which, if successful, might bring time resolution down to 10^{-6} seconds or better.

VIII. Purification of Photo-catalyst P700

We have undertaken some renewed attempts to further purify P700. Starting from spinach and blue-green algae, we used various organic solvents for primary extractions to remove as much lipid material as possible, we have worked with acetone and other more and less polar solvents to differentially remove the bulk chlorophyll over P700. We have surveyed a number of deficient pigment algae mutants and although several of these did not show any P700 at all, we have not yet come across one which showed a particularly rich amount of it. We have investigated a number of leaves some of which were very pale, but none of these showed a higher than normal content of P700. Also the use of Urtica and Helianthus leaves, rich in chlorophyllase, showed no advantage. We still feel the problem is important enough to be pursued in the future and are convinced that sooner or later we will find a natural material or a mutant much enriched in P700.

IX. Fluorescence Studies

One of our studies concerned the fluorescence yield as a function of intensity at room temperature in fresh chloroplasts. In such chloroplasts there is a distinct "competition" between the reduction of substrate and loss of light as fluorescence, i.e., light, if it cannot be used in photochemistry, can escape partly as fluorescence. Fluorescence, therefore, is a good indicator of electron transport. It was proposed earlier (1952) by Duysens that in green cells fluorescence originates mainly from pigment connected to the second photoreaction. Though our data grossly confirmed this statement, we were puzzled by its possible significance. One of the reasons for this is that purple sulfur bacteria, which do not seem to have a second photoact in the same sense as green plants, do fluoresce and the intensity is anti-parallel with substrate reduction.

Emission and excitation studies in samples of various algae and chloroplasts at the temperature of liquid nitrogen appear to yield at least partial answers. We have come across an undescribed fluorescence band located at 698 mu, which occurs only at very low temperatures ($< -150°C$). This emission proved to be of particular interest because even at this low temperature it required light to be activated, meaning that a true photochemical event is being observed. Presently, we are making quantitative studies of this "detrapping" at low temperature and trust this will yield important information concerning quantum flow and pigment interaction.

PUBLICATIONS (1962-1963)

Kok, B., Cooper, B. and Yang, L. "Sensitization of Chloroplast Reactions. I. Sensitization of Reduction & Oxidation of Cytochrome C by Chloroplasts", Plant Physiol., 38, 274-279 (1963).

Kok, B., Cooper, B., and Yang, L. "Electron Transport in Chloroplast Reactions", In: Microalgae and Photosynthetic Bacteria, Plant and Cell Physiol. 373-396 (1963).

Hoch, G., Owens,O.v.H., and Kok, B. "Photosynthesis and Respiration", Arch. Biochem. Biophys., 101, 171-180 (1963).

Hoch, G., and Kok, B. "A Mass Spectrometer Inlet System for Sampling Gases Dissolved in Liquid Phases", Arch. Biochem. Biophys., 101, 160-170 (1963).

Kok, B. "Significance of P700 as an Intermediate in Photosynthesis", Proceedings of the Fifth Internat. Congressof Biochemistry, Pergamon Press, VI, 73-81 (1963).

Hoch, G., and Martin, I. "Photo-Potentiation of Adenosine Triphosphate Hydrolysis", Biochem. Biophys. Res. Comm., 12, 223-228 (1963).

Hoch, G., and Martin, I. "Two Light Reactions in TPN Reduction by Spinach Chloroplasts", Arch. of Biochem. Biophys. 102, 430-438 (1963).

Owens, O.v.H., and Hoch, G. "Enhancement and De-Enhancement Effect in Anacystis Nidulans", Biochim. Biophys. Acta, 75, 183-186 (1963).

Govindjee, Owens, O.v.H., and Hoch, G. "A mass-spectroscopic study of the EMERSON enhancement effect", Biochim. Biophys. Acta, 75, 281-284 (1963).

Govindjee, Govindjee, and Hoch, G. "The Emerson Enhancement Effect in TPN-Photoreduction by Spinach Chloroplasts", Biochem. Biophys. Res. Comm., 9, 222-225 (1962).

Kok, B. "Light Conversion in Photosynthesis", Biologistics for Space Systems Symposium, pp. 83-104 (1962).

Zill, L. P., and Cheniae, G. M. "Lipid Metabolism", Plant Physiol. 13, 225-264 (1962).

PUBLICATIONS IN PRESS (1962-1963)

Kok, B., and Hoch, G. "The Photoreactions of Photosynthesis", Proceedings of Symposium on Photosynthesis, Paris, 1962.

Hoch, G., and Owens, O.v.H. "Oxygen Metabolism in Anacystis Nidulans", Proceedings of Symposium on Photosynthesis, Paris, 1962.

Kok, B., Rurainski, H. and Harmon, A. "Photo-Oxidation of Cytochromes c, f and Plastocyanin by Detergent Treated Chloroplasts", Plant Physiol., 1963.

Kok, B. "Fluorescence Studies", Symp., Airlie House, 1963.

Kok, B. "Photosynthetic Electron Transport", Symp., Airlie House, 1963.

Hoch, G., and Owens, O.v.H. "Photoreactions and Respiration", Symp., Airlie House, 1963.

Beinert, H., and Kok, B. "Relationship between Light Induced EPR Signal and Pigment P700", Symp., Airlie House, 1963.

Cheniae, G. "Fatty Acid Synthesis by Extracts of Euglena I", Biochim. Biophys. Acta, 1963.

Cheniae, G. "Fatty Acid Synthesis by Extracts of Euglena II", Arch. Biochem.,1963.